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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: DAHM *et al.*

Serial No.: 09/601,645
Confirmation No.: 7793

Filed: August 4, 2000

For: METHOD FOR THE QUANTITATIVE DETERMINATION OF TUMOR CELLS IN A BODY FLUID AND TEST KITS SUITABLE THEREOF

Art Unit: 1634

Examiner: Goldberg, J.A.

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The following attachment is provided:

(1) Executed Declaration of Michael W. Dahm Pursuant to §1.132

OIPE JC67
MAR 08 2004
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	DAHM *et al.*		
Serial No.:	09/601,645	Art Unit:	1634
Filed:	August 4, 2000	Examiner:	Goldberg, J.A.
Conf. No.:	7793	Customer No.:	24961

For: *METHOD FOR THE QUANTITATIVE DETERMINATION OF TUMOR CELLS IN A BODY FLUID AND TEST KITS SUITABLE THEREOF*

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Michael W. Dahm , declare as follows:

1) I am an inventor of and am familiar with the subject matter of the above-captioned application, which was filed on August 4, 2000.

2) I received M.D. degrees from the Medical University of Messina (Messina, Italy) in November 1986 and from the Technical University of Munich (Munich, Germany) in September 1994. I have held post-doctoral research fellowships in the Department of Laboratory Medicine and the Department of Rheumatology and Immunology at the Medical University of South Carolina in Charleston, S.C. and in the Department of Virology at the Paul Ehrlich Institute in Langen, Germany. I have also completed residencies in internal medicine at the Ludwigs-Maximillian University of Munich, München-Bogenhausen, Germany, and in dermatology at the Technical Institute of Munich in Munich, Germany and the Medical University of Florence in Florence, Italy.

3) I am currently General Manager at HEXAL Gentech ForschungsGmbH, located in Holzkirchen, Germany. I have held this position since 1998.

4) My research interests include cell biology and cancer biology. I have worked on developing strategies for specific enrichment of circulating and/or metastatic tumor cells from body fluids such as blood and urine.

5) In my capacity as a researcher, myself and other scientists have demonstrated that by using a density centrifugation-based method with cell

separation medium in the density range of 1.060-1.065 g/ml, disseminated tumor cells in a body fluid can be specifically concentrated, detected and quantitated without (i) significant contamination from telomerase-positive non-tumor cells; or (ii) significant loss of tumor cells from the body fluid sample.

6) The results shown below demonstrate that, by using a cell separation medium with a density in the range of 1.060-1.065 g/ml, one can specifically concentrate and quantitate disseminated tumor cells in a body fluid, such as blood or urine. This density range of 1.060-1.065 g/ml allows specific concentration of tumor cells, without significant contamination from telomerase-positive non-tumor cells. Using a cell separation medium density between 1.060 g/ml and 1.065 g/ml allows the catalytic subunit of telomerase (hTRT) to be used as a specific, ubiquitous tumor-associated marker for the detection and quantification of circulating solid tumor cells in human body fluids. As demonstrated below, a cell separation medium density below 1.060 g/ml leads to significant loss of tumor cells from the concentrated tumor cell fraction. Further, a cell separation medium density greater than 1.065 g/ml leads to contamination of the concentrated tumor cell fraction with telomerase positive non-tumor cells.

I. Employing a cell separation medium density of at least 1.060 g/ml is necessary for preventing significant loss of tumor cells concentrated from a body fluid sample

The following experiments were performed to determine the optimal cell separation medium density for enriching tumor cells in a body fluid sample, while avoiding significant loss of tumor cells. Specifically, these experiments demonstrate that a cell separation medium density of about 1.060 g/ml is the lower limit of the density range for optimal recovery of tumor cells from a body fluid when isolating the tumor cells using a density centrifugation-based method.

Methods:

A discontinuous Percoll density gradient ranging from 1.020 g/ml up to 1.080 g/ml was created by layering cushions of decreasing densities, one on top of each other, to create seven different density layers (1.02 g/ml, 1.03 g/ml, 1.040 g/ml, 1.050 g/ml, 1.060 g/ml, 1.070 g/ml and 1.080 g/ml represented layers 2-8, respectively) and T289 cells (T289/PBS) layered on top of the 1.02 g/ml layer (layer 1). The Percoll cell separation medium was adjusted to the desired density according to the instructions provided by the manufacturer and as described in the specification (see, *e.g.*, page 18, line 24 through page 19, line 16). Cells of the melanoma cell line T289 were cultured according to the instructions provided by the supplier (American Type Culture Collection; ATCC) and harvested as described in the specification (see, *e.g.*, page 31, line 25-32). Briefly, cells were grown to confluence, trypsinized and washed in cell culture medium. An aliquot of cells was stained with trypan blue to determine the number of viable cells in the sample. Approximately 1000 cells suspended in PBS were carefully layered on top of the upper 1.020 g/ml density cushion and centrifuged at 1000 x *g* for 30 min at 4°C. After centrifugation, each layer was carefully removed and processed for RNA extraction and RT-PCR using primers specific for the catalytic subunit of telomerase (hTRT) or actin (positive control) as described in the specification (see, *e.g.*, page 34, line 28 through page 36, line 19). To quantify the RT-PCR amplicons from each density layer, each band was individually imported into the Adobe photoshop software (Macintosh OSX, Version 7.0.1) and mean values were determined by standard methods.

Results:

RT-PCR amplicons for hTRT were strongest in density layers 3 (1.030 g/ml), 5 (1.050 g/ml) and 6 (1.060 g/ml). Based on the mean values determined for each density layer, 94% of hTRT positive cells banded at a density of 1.060 g/ml or lower. Therefore, using a density of at least about 1.060 g/ml will result

in enrichment of nearly all melanoma cells (*i.e.*, all melanoma cells banding at a density of 1.060 g/ml or lower) without significant tumor cell loss.

Similar experiments were performed using a variety of different tumor cell lines, including LNcap (prostate carcinoma), SKBr3 (mammary carcinoma), HT-29 (colorectal adenocarcinoma), A549 (lung carcinoma), SK-Hep-1 (liver adenocarcinoma), HeLa (cervical carcinoma) and PC3 (prostate cancer). The results demonstrate that density centrifugation using a cell separation medium with a density of about 1.060-1.070 g/ml results in enrichment, without significant loss, of tumor cells from a fluid sample.

II. Employing a cell separation medium density of 1.065 g/ml or less prevents contamination of the enriched tumor cell fraction with telomerase-positive non-tumor cells from a body fluid sample

Results from the previous section demonstrate that a cell separation medium density of about 1.060-1.070 g/ml is necessary for enrichment of a tumor cell fraction from a body fluid, while avoiding significant tumor cell loss. The following experiments show that using a cell separation medium of about 1.065 g/ml or less prevents contamination of the enriched tumor cell fraction with telomerase-positive non-tumor cells.

Activated lymphocytes and hematopoietic stem cells present in human peripheral blood possess active telomerase and consequently express the catalytic subunit of human telomerase (hTRT) as well as the RNA component of human telomerase (hTR). Therefore, these cell populations represent telomerase-positive non-tumor cells present in the body fluid blood. Since this expression is not disease-specific and therefore detectable in healthy individuals as well as in cancer patients, a technology that depletes telomerase-positive non-tumor cells in blood is necessary to allow the specific detection of circulating cancer cells in a peripheral blood sample. The following experiments were designed to determine the optimal cell separation medium density for preventing contamination from telomerase-positive non-tumor cells in a body

fluid sample. Specifically, the experiments described below define about 1.065 g/ml as the upper limit of the density range for preventing such contamination.

A. Depletion of hTRT-positive non-tumor cells from peripheral blood using a cell separation medium density of 1.065 g/ml

The following experiments were performed to determine an appropriate range of cell separation medium density sufficient to deplete activated lymphocytes (*i.e.* telomerase-positive non-tumor cells) from peripheral blood.

Methods:

Peripheral blood (20 ml) was drawn from ten healthy volunteers after obtaining consent and used immediately in the following experiments. For hTRT analysis, the collected peripheral blood from each volunteer was divided into two 10 ml aliquots and centrifuged individually on a Percoll cell separation medium with a density of either 1.070 g/ml or 1.065 g/ml. For fluorescence activated cell sorting (FACS) analysis, the collected peripheral blood from one volunteer was divided into two 10 ml aliquots and centrifuged individually on a Percoll cell separation medium with a density of 1.065 g/ml or a Ficoll cell separation medium with a density of 1.077 g/ml. The cell separation medium was adjusted to the desired density according to the instructions provided by the manufacturer or as described in the specification (see, *e.g.*, page 18, line 24 through page 19, line 16). The centrifugation procedure was performed as described in the specification (see, *e.g.:*, page 30, lines 33-39). Briefly, 10 ml of each blood sample was carefully layered on top of each density preparation (1.065 g/ml, 1.070 g/ml or 1.077 g/ml) and centrifuged at 1000 x *g* for 30 min at 4°C, with slow acceleration and no brake.

After centrifugation, the upper part of the supernatant was discarded and the interphase, together with the remaining supernatant, was collected. The recovered cell fraction was washed in PBS and processed for either fluorescence activated cell sorting (FACS) to detect CD45 positive mononuclear cells or RT-PCR to detect hTRT expression. For FACS analysis, cells were labeled with a

PE-labeled pan-leukocyte monoclonal antibody against CD45 (31264X, Pharmingen, San Diego, CA, U.S.A.) as described by the manufacturer. An aliquot of 10^4 cells was subsequently analyzed by FACS (BD FACSCalibur, Beckton Dickinson, N.J., U.S.A.) using standard procedures. Cell lysis, RNA extraction, RT-PCR using hTRT-specific primers, agarose gel analysis, visualization with ethidium bromide and photo documentation were performed by methods as described in the specification (see, *e.g.*, page 6, line 12 through page 9, line 31; page 34, line 28 through page 36, line 19).

Results:

FACS analysis of peripheral blood from healthy donors (*i.e.*, blood containing no tumor cells) showed a distinct CD45-positive cell population (mononuclear cells, such as lymphocytes and monocytes, some of which are telomerase-positive non-tumor cells) when the blood was concentrated by centrifugation at a density of 1.077 g/ml. In contrast, when peripheral blood was centrifuged at a density of 1.065 g/ml, the CD45 positive mononuclear cells were almost entirely depleted.

hTRT-specific RT-PCR analysis of the peripheral blood samples enriched using a density of 1.070 g/ml demonstrated that 30% of healthy donors tested positive for hTRT expression. This result indicated that when a cell separation medium with a density of 1.070 g/ml is used, there is significant contamination of telomerase-positive non-tumor cells at the interphase. In contrast, all healthy donor samples tested negative for hTRT when cells were concentrated using a density of 1.065 g/ml.

The above results demonstrate that the interphase obtained by centrifugation of peripheral blood using a cell separation medium density of 1.065 g/ml contains no telomerase positive mononuclear cells (*i.e.* hTRT positive non-tumor cells). This is evidenced by (i) thorough depletion of a FACS-detectable CD45 positive mononuclear cell population at the interphase in comparison to Ficoll at a density of 1.077 g/ml; and (ii) absence of detectable

hTRT expression from the interphase. A cell separation medium density of greater than 1.065 g/ml, while avoiding significant loss of tumor cells, would result in significant contamination of the enriched cell fraction with telomerase-positive non-tumor cells. Therefore, these results, taken in conjunction with the results provided in Section I, demonstrate that using a cell separation medium density in the range of 1.060-1.065 g/ml to concentrate tumor cells from a body fluid, is optimal for depleting telomerase-positive non-tumor cells while avoiding excessive loss of tumor cells from the concentrated tumor cell fraction.

B. (i) Depletion of hTRT-positive non-tumor cells from a blood contaminated urine sample

The following experiments were conducted to investigate whether the combination of density centrifugation and subsequent hTRT specific RT-PCR is capable of detecting exfoliated tumor cells in urine. Exfoliated tumor cells, typically in conjunction with blood contamination, are often found in the urine of patients suffering from bladder cancer. A model system consisting of urine and blood from healthy donors was used to mimic that condition and to determine the cell separation medium density required to deplete hTRT positive mononuclear cells.

Methods:

Percoll with a density of 1.070 g/ml, 1.060 g/ml, 1.050 g/ml or 1.040 g/ml was adjusted as described above. Blood and urine were obtained from healthy volunteers (*i.e.*, containing no tumor cells in the body fluids) after obtaining consent and used immediately in the following experiments. The collected donor urine (38 ml) was spiked with 2 ml of donor blood and four equal 10 ml aliquots were prepared. Each 10 ml aliquot was mixed with 30 ml PBS to yield a total of volume of 40 ml. This volume was carefully poured on top of cell separation media prepared at each density. The centrifugation procedure, isolation of the recovered cell fraction, cell lysis, RNA extraction, RT-PCR, agarose gel analysis, visualization with ethidium bromide and photo

documentation were performed as described above and in the specification (see, *e.g.*, page 30, line 33 through page 31, line 14; and page 34, line 28 through page 35, line 28). RT-PCR was performed using hTRT-specific primers and carcinoembryonic antigen (CEA)-specific primers. CEA was used as a positive control for detection of blood cells.

Results:

No hTRT-specific amplicons were detectable at densities of 1.040 g/ml, 1.050 g/ml and 1.060 g/ml. hTRT-specific amplicons were, however, detected at a density of 1.070 g/ml. The positive control, CEA, was detectable at all densities, confirming the presence of blood cells. These results demonstrate that a density of under 1.070 g/ml is necessary in order to deplete hTRT positive non-tumor cells from a blood contaminated urine sample.

B. (ii) Enrichment of hTRT-positive tumor cells from a blood contaminated urine sample

The following experiments were conducted to investigate whether a density of 1.060 g/ml was capable of enriching hTRT positive tumor cells from a blood contaminated urine sample. Therefore, urine and blood from a healthy donor was collected and spiked with tumor cells from a prostate cancer cell line.

Method:

The methods used in this experiment are identical to the methods described above in B(i) for spiking of human peripheral blood into urine, with the exception that Percoll was used at a single density of 1.060 g/ml and different numbers of cells (20, 40 and 10^5) of the prostate carcinoma cell line DU45 were spiked into the blood/urine sample. The DU45 cell line was cultured as described by the supplier (ATCC). After centrifugation of the tumor cell-spiked blood/urine samples on 1.060 g/ml density medium, cells at the interphase were collected and harvested as described in the specification (see, *e.g.*, page 31, line 25-32). RT-PCR was performed using primers specific for hTRT, CK19 (cytokeratin 19; expressed in blood cells), CK20 (cytokeratin 20; expressed in

epithelial cells) or PSA (prostate specific antigen). PSA, which is not expressed in DU45 cells, was used as a negative control.

Results:

Unspiked blood/urine samples were hTRT-negative, demonstrating that the hTRT-positive mononuclear cells were depleted from the sample. Increasing amounts of spiked tumor cells resulted in increasing amplification of hTRT transcripts. CK19 was detected in all samples, demonstrating the presence of blood cells in each sample. CK20 was only detected when higher numbers of DU45 cells (*i.e.* 10^5) were spiked into the sample. Prostate specific antigen (PSA) was not detected in any sample since the prostate cancer cell line DU45 does not express PSA.

These results, and the results described above, demonstrate that a density of about 1.060 g/ml is sufficient to both reproducibly deplete hTRT positive non-tumor cells and reproducibly enrich hTRT-positive tumor cells spiked into a blood contaminated urine sample. Thus, the detection of tumor cells using hTRT as a surrogate molecular tumor associated marker is possible in body fluids such as blood and urine.

III. Conclusions

The above experiments demonstrate that:

(1) a density-based centrifugation method with a cell separation medium density range of between 1.060 g/ml to 1.650 g/ml depletes telomerase positive non-tumor cells from the concentrated tumor cell fraction obtained from body fluids, such as peripheral blood or urine, while avoiding a significant loss of tumor cells from the fraction;

(2) the density range from 1.060 g/ml to 1.065 g/ml enriches and allows detection and quantification of a variety of disseminated tumor cells stemming from different tumors such as breast cancer, cancers of the gastrointestinal tract (*e.g.*, colorectal cancer), prostate cancer, lung cancer, cervical cancer and malignant melanoma; and

(3) the catalytic subunit of human telomerase (hTRT) can be employed as a tumor associated surrogate marker when tumor cells from a body fluid are enriched using a cell separation medium density ranging from 1.060 g/ml to 1.065 g/ml.

Thus, concentration of circulating tumor cells from a body fluid using density-based centrifugation with a cell separation medium of density of 1.060-1.065 g/ml permits quantification of the tumor cells. Any density below about 1.060 g/ml will lead to losses of tumor cells from the enriched tumor cell fraction and an underestimation of the number of tumor cells in the fraction. On the other hand, a cell separation medium density greater than about 1.065 g/ml will result in enrichment of hTRT positive mononuclear cells (telomerase positive non-tumor cells) from the body fluid and generate false positive results, *i.e.*, an overestimate of the number of tumor cells in the fraction.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

M. Dahm

Michael W. Dahm

Date 17.02.2004